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Transgenic Mouse Model for the Fragile X Syndrome

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Transgenic fragile X knockout mice have been constructed to provide an animal model to study the physiologic function of the fragile X gene (FMR1) and to gain more insight into the clinical phenotype caused by the absence of the fragile X protein. Initial experiments suggested that the knockout mice show macroorchidism and cognitive and behavioral deficits, abnormalities comparable to those of human fragile X patients. In the present study, we have extended our experiments, and conclude that the Fmr1 knockout mouse is a reliable transgenic model to study the fragile X syndrome. © 1996 Wiley-Liss, Inc.

KEY WORDS: fragile X syndrome, Fmr1 knockout mice, transgenic animal model, spatial learning, macroorchidism, hyperactivity

INTRODUCTION

The fragile X syndrome is caused by transcriptional inactivation or deletion of the fragile X gene (FMR1) [Verkerk et al., 1991; Pieretti et al., 1991; Willems, 1994; Kooy et al., 1996]. Absence of FMRP, the protein product of FMR1 [Verheij et al., 1993], results in the fragile X phenotype, consisting of mental retardation, characteristic facial features, macroorchidism, and behavioral abnormalities [Hagerman, 1991]. Despite the fact that the FMR1 gene was cloned 5 years ago, little is known about the physiologic function of FMRP. The discovery of a unique patient with a missense mutation in the FMR1 gene [De Boulle et al., 1993] led to the identification of RNA binding domains in FMRP, and in vitro RNA binding properties of FMRP was subsequently demonstrated [Ashley et al., 1993; Siomi et al., 1993; Verheij et al., 1995]. Recently, two autosomal genes, FXR1 and FXR2, have been identified that share a high sequence homology with FMR1, especially in the 5' and middle region of the gene, including the two RNA binding KH domains [Siomi et al., 1995; Zhang et al., 1995]. The three proteins are located in the cytoplasm, are able to bind their own RNA as well as a set of unknown RNAs, and associate tightly with themselves and with other proteins in the cell [Zhang et al., 1995].

However, the precise function of FMRP and its homologues is still unknown, and clues about the mechanisms that cause the abnormalities observed in fragile X syndrome are missing. To gain more insight in these physiologic and pathologic processes, we have constructed fragile X knockout mice [Bakker et al., 1994]. The knockout mice were generated by replacing the wild type murine Fmr1 gene by a nonfunctional Fmr1 gene with an interrupting neomycin cassette in exon 5, using homologous recombination in embryonic stem cells. As a result of this gene "knockout," mutant mice are no longer able to make stable Fmr1 mRNA or protein. Although the knockout mutation in the transgenic animal model is different from the mutation found in human fragile X patients, both mutations lead to a loss-of-function of the FMR1 gene and, therefore, absence of FMRP.

Initial experiments showed that the knockout mice are perfectly viable and fertile, without any obvious phenotypic or pathologic abnormality. However, they show macroorchidism, learning deficits, and hyperactivity [Bakker et al., 1994; Willems et al., 1995]. In the present study, we further evaluated the phenotype of the fragile X knockout mice.

MATERIALS AND METHODS

Animal Handling

Only male knockout mice and their normal male littermates were used. Male knockout mice were kept together in the same cages as their normal male littermates on a 12 h light:dark cycle. Food and water were supplied ad libitum. All experiments were performed

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without knowledge of the genotype of the mice that were tested.

Animal Dissection

All mice were killed by ether anesthesia and dissected. Both testes were removed, cleaned and weighted together, taking care not to include any additional non-testicular tissue. Brain, kidney, heart, spleen, and liver were carefully prepared free, cleaned, and weighed.

Morris Water Maze

The mice were trained to find a platform (∅ 15 cm) in a round basin (∅ 150 cm) filled with water (18°C) made opaque by adding nontoxic opacified Lytron 621, exactly as described before [Bakker et al., 1994]. The platform was placed 1 cm underneath the surface. The interior of the experimental room was not changed during the experiments. Albino mice were excluded from the experiments, as their vision is often impaired. The mice first learned to find the platform in its hidden position during 12 training trials. Each trial consisted of 4 releases from 4 different positions at the side of the pool (N, E, S, and W position). The maximum time the mice were allowed to swim was 2 min, after which time mice were put on the platform and were left there for 15 sec. The total swimming time of the four releases is called the escape latency. After the 12 learning trials, the platform was placed at the center of the opposite quadrant and 4 reversal trials were performed exactly as in the learning trials.

Probe trials were performed after the 8th learning trial and after the 4th reversal trial. In this test, the time the mice swam in the quadrant the platform had been removed from (target quadrant), was registered for 100 sec.

E-Maze

Mice were released at the tip of the middle arm of an E-shaped basin filled with water. An escape platform was placed initially at the tip of the left arm. The mice were trained to locate this platform during 5 days, with three trials each day. After the training sessions, the escape platform was moved to the right arm, and the mice were subjected to the same testing procedure. Both the time needed to reach the platform (escape latency), and the amount of errors were registered manually for each trial. An error is registered each time a mouse swims into any arm of the E-shaped basin without escaping to the platform, after its release from the tip of the middle arm.

RESULTS

Organ Weight

Brain, kidney, heart, spleen and liver from 9 control and 11 knockout mice were weighted at the age of 8-10 weeks. No significant difference was observed for any of the tissues (Student's t test, $P > 0.5$). Light microscopic examination of all these tissues was normal [Bakker et al., 1994].

Testicular weight was measured in various age groups of mice from 7 to 60 weeks. The combined testicular weight increased with age till the mice were about 23 weeks, and the weight started to decrease from that age on (Fig. 1). In each age group, the testes of the knockout mice (Table I) were significantly heavier than those of their normal littermates (Student's t test, $P < 0.05$ at 40–50 days, $P < 0.001$ in all other age groups). Seventy-eight percent of the recorded weights from mice from 40 to 50 days were in the overlapping zone of the two genotypes. The overlap decreased to 56% and 16% in the age groups from 60–70 and 90–100 days respectively. No overlap in weight between knockout and control mice existed at 168–170 days of age, when the testicular weight was at its maximum in both groups of mice. Thereafter, the overlap increased again to 11% and 52% in the age groups from 300–320 and 370–400 days, respectively.

TABLE I. Combined Testicular Weight ± SD of Knockout and Control Mice at Different Ages

Age	Control mice	Knockout mice
40–50 days	168 ± 23 (n = 22)	190 ± 33 (n = 19)
60–70 days	196 ± 24 (n = 19)	239 ± 24 (n = 21)
90–100 days	218 ± 19 (n = 11)	280 ± 26 (n = 16)
168–170 days	246 ± 21 (n = 10)	314 ± 16 (n = 7)
300–320 days	217 ± 19 (n = 20)	263 ± 20 (n = 17)
370–400 days	186 ± 33 (n = 26)	249 ± 34 (n = 26)

Morris Water Maze Test

In the initial tests, 14 knockout mice were compared with 11 controls in the Morris water maze [Bakker et al., 1994]. To further evaluate this effect, we have compared an additional 22 knockout and 17 control mice, using exactly the same protocol as in the initial test. The two sets of data were pooled. Both the 36 knockout and the 28 control mice learned to find the hidden escape platform during the 12 training trials (Fig. 2, ANOVA, $P < 0.0001$). The knockout mice needed more time to reach the escape platform over 12 days as the effect of the genotype was significant (ANOVA, $P = 0.0007$). No difference in the rate of learning to find the escape platform was apparent, as the interaction of genotype versus trial was not significant (ANOVA, $P > 0.5$). That the mice are able to learn to find the escape platform was confirmed in the first probe trial, a trial performed in the absence of the escape platform after the 8th training session. Control mice swam 47.1 ± 2.7 % (mean ± SEM) of the total swimming time in the quadrant which previously contained the platform, and knockouts 47.5 ± 2.3 %. These percentages are statistically different from the expected 25% due to chance alone (one sample t test, $P < 0.001$ for each genotype), illustrating that both groups of mice are perfectly able to learn the test. No difference between the controls and the knockouts was apparent in the probe trial (Student's t test, $P > 0.5$).

During the reversal trials, when the platform is placed in the opposite quadrant as compared to the learning trials, both types of mice needed more time to find the platform in its new position, especially on the

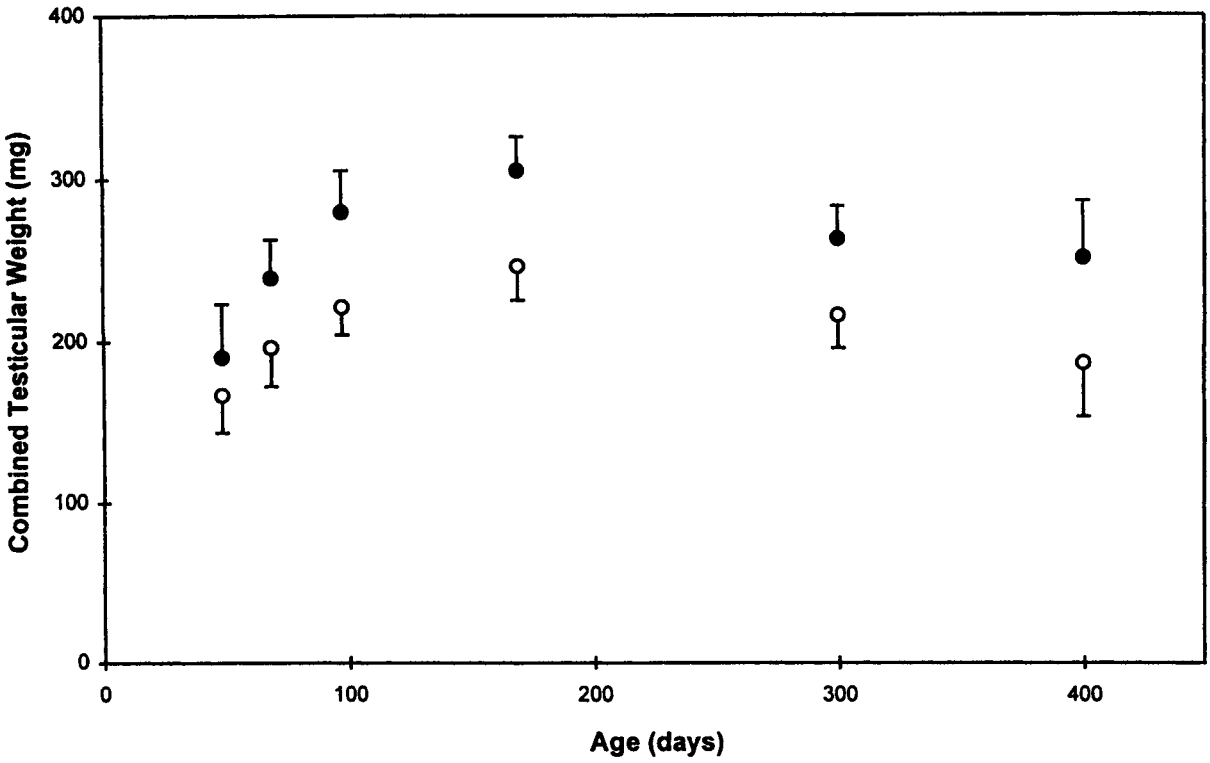

Fig. 1. Combined testicular weight (mean ± SD) of knockout (●) and control (○) mice in six different age groups.

first day, but significantly improved their performance over 4 days (ANOVA, $P < 0.0001$). There was a significant effect of genotype on the escape latency (ANOVA, $P < 0.0001$). The interaction effect of genotype versus trial was also significant (ANOVA, $P = 0.024$). This indicates that the knockout mice needed more time over the four reversal trials to escape to the platform. The difference in learning performance between knockout and control mice was greater during reversal trials than during learning trials (see asterisks indicating significance per day in Fig. 2).

A second probe trial performed after the last reversal trial confirmed that both groups were perfectly able to learn the test, but did not reveal any difference between knockouts and controls. Knockouts swam 38.9 ± 1.8% (mean ± SEM) of their time in the quadrant the platform had been removed from, as compared to 43.3 ± 2.0 % for control mice (Student's t test, $P > 0.1$).

E-Maze Test

The performance in the E-maze task of 19 knockout mice, aged 32–34 weeks, was compared with that of 23 control littermates. In this task, the mice have to find a visible escape platform, located in a fixed position in an E-shaped basin filled with water. Both escape latency and amount of errors were scored. Both groups of mice learned to find the escape platform during 5 days of learning sessions (ANOVA, $P < 0.0001$). Control mice made only 0.7 ± 0.3 errors (mean ± SE) on day 5 as compared to 2.6 ± 0.4 errors on day 1, whereas knockout mice made 1.0 ± 0.7 errors on day 5 as compared to 2.6 ± 0.5 errors on day 1. The genotype had no effect on the amount of errors (ANOVA, $P > 0.5$), indicating that knockout littermates learned the task as well as the controls. Also, the interaction of genotype versus trial was not significant (ANOVA, $P > 0.5$).

After the training sessions, the escape platform was placed in the opposite arm of the E-maze. The mice made a high number of errors on the first day (controls: 15.2 ± 1.9, knockouts 19.3 ± 2.6) of the reversal trials, but learned to find the platform in its new position during the reversal trials (ANOVA, $P < 0.0001$), making only 0.8 ± 0.3 (controls) and 1.3 ± 0.5 (knockouts) errors on day 5. The knockout mice performed the reversal test not significantly different from the control mice, as the genotype had no effect on the amount of errors (ANOVA, $P = 0.29$), and also the interaction of genotype versus trial was not significant (ANOVA, $P = 0.16$).

Similar results were obtained when the escape latency was analyzed instead of the amount of errors.

DISCUSSION

Although the knockout mutation in transgenic fragile X mice is different from the mutations causing fragile X syndrome in humans (CGG repeat amplification or deletion), Fmr1 knockout mice resemble human fragile X patients in having no Fmr1 protein. Therefore, we anticipated that the knockout mice might be a good animal model for fragile X syndrome. The three most prominent characteristics of the fragile X syndrome are macroorchidism, mental retardation, and

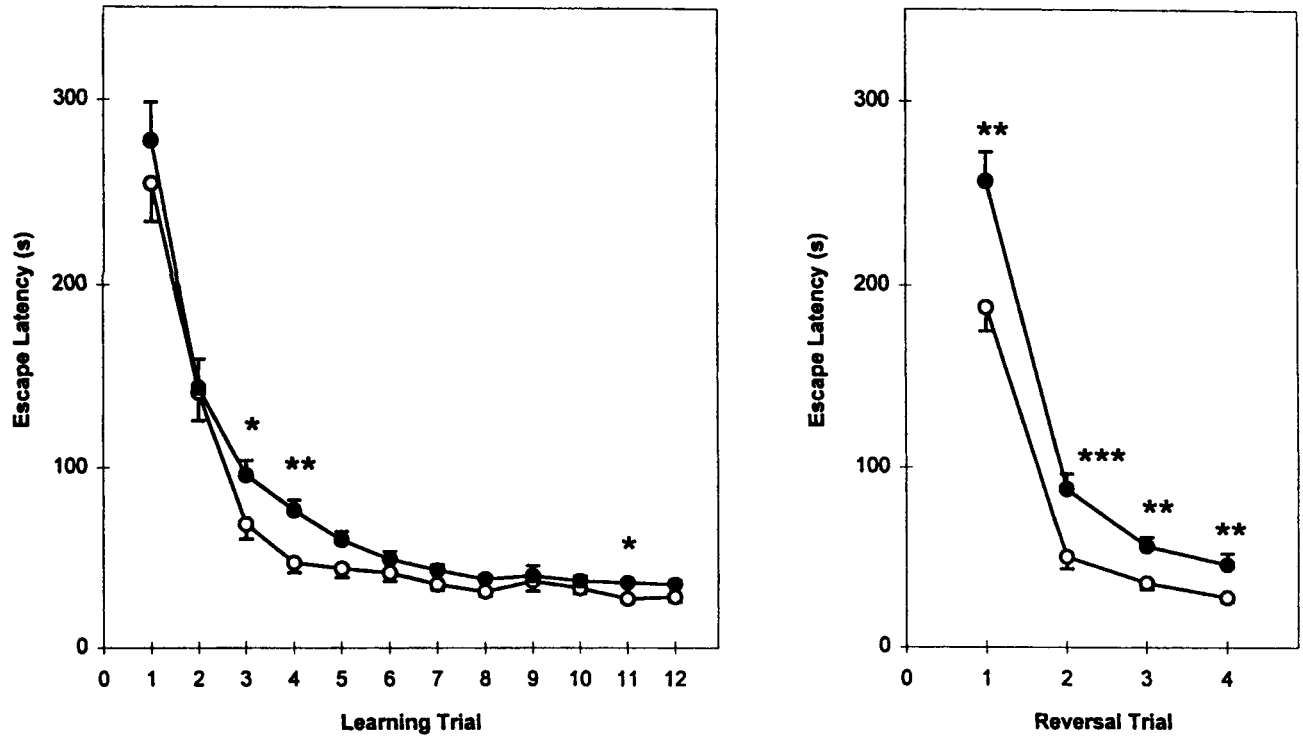

Fig. 2. Combined Morris water maze tests results (mean ± SEM) of the initial study by Bakker et al. [1994] and the present study. Escape latency of knockout (●) and control (○) mice was analyzed in learning trials and reversal trials. ⋆ : $0.01 < P < 0.05$; ⋆⋆: $0.001 < P < 0.01$; ⋆⋆⋆: $P < 0.001$ (post-hoc t test).

behavioral abnormalities such as hyperactivity and autistic features. Our initial study of the knockout mice indicated that also the knockout mice had macroorchidism, and cognitive and behavioral abnormalities [Bakker et al., 1994]. In the present study we expanded our data on macroorchidism and cognitive function abnormalities in the knockout mice, and completely confirmed our initial findings.

Similar to male human patients, fragile X knockout mice developed progressive macroorchidism with time, and a maximum increase of 34% in testicular weight was found in knockouts of 168–170 days. Thereafter, the macroorchidism became less. A decrease in macroorchidism has also been observed in patients at high age. Light microscopic examination of knockout testes revealed no pathological abnormalities. However, also in testes from human fragile X patients no consistent light microscopic abnormalities have been found. Therefore, the pathogenic mechanism leading to macroorchidism in the fragile X syndrome remains unclear. Both abnormal concentrations of a trophic factor, as well as intrinsic testicular abnormalities could be present.

Initial motor activity tests on a limited number of mice (five knockout and five control mice of 150 days of age) showed that male knockouts made significantly more photocell beam interruptions over a 40 min interval than male controls [Bakker et al., 1994]. To study larger groups of mice, we rebuild the motor activity tracker. In the new apparatus, the observed number of photocell beam interruptions was 5–10-fold higher than in the original apparatus, and differences in motor activity between various age groups of knockout and control mice were smaller than observed in the original test, or even completely absent. As we have no explanation for the differences observed between the original experiment and the present experiments, we plan to rebuild the original motor activity tracker to investigate the motor activity of fragile X knockout mice further.

Cognitive function of knockout and control mice was studied in the Morris water maze. In the learning trials, both groups of mice learned to find the escape platform during 12 training trials, which was confirmed by the probe tests. Although the differences in escape latency were small, mutant mice consistently needed more time to locate the platform, resulting in a high overall significance over the 12 learning trials (ANOVA, $P < 0.0001$). Interaction of genotype versus trial was never significant, indicating that knockout mice learn at the same rate as control mice. In the reversal test, both groups of mice initially needed more time to locate the platform in the opposite quadrant of the pool than in the last trials of the learning test, but both groups of mice learned to find the escape platform during four trials. The knockout mice needed more time to reach the escape platform than the controls, both in the initial [Bakker et al., 1994] and the present experiments. Impaired performance of the knockout mice when compared with the control mice was more pronounced during the reversal than during learning of the task, as

evidenced by the significance of the statistical analysis by Student's t test (Fig. 2). The impairment in the reversal of the Morris water maze is unlikely to be the result of a general failure to switch from one set of learned responses to another due to increased perseveration, as knockout mice performed the E-maze task as well as the control group. The lesser performance of the knockout mice during learning and reversal trials in the Morris water maze is therefore probably caused by deficits in spatial memory [Morris, 1981, 1984]. Spatial orientation is the ability to find an invisible object dependent on distal cues, as tested in the Morris water maze but not in the E-maze test. Visual-spatial disabilities have also been reported in fragile X patients [Cianchetti et al., 1991], suggesting a possible involvement of the hippocampus, a brain structure involved in learning and memory, in fragile X syndrome [Morris et al., 1982; Squire, 1992]. The visual-spatial disabilities of the knockout mice led us to investigate experimentally whether altered long-term potentiation (LTP) is perhaps involved in the fragile X syndrome. However, no evidence suggesting the involvement of FMR1 in LTP could be found [see accompanying paper by Godfraind et al., 1996].

Like fragile X patients, the knockout mice are perfectly viable, have normal fertility, and show no obvious pathologic or microscopic abnormalities, not even in the brain or testes [Bakker et al., 1994; Willems et al., 1995]. In conclusion, we think therefore that a faithful transgenic mouse model for the fragile X syndrome has been developed.

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